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Age-dependent decrease of retinal kynurenate and kynurenine aminotransferases in DBA/2J mice, a model of ocular hypertension

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Abstract

The study examines age-dependent changes of kynurenic acid (KYNA) content and kynurenine aminotransferases (KAT I and KAT II) celluar expression in the retinas of DBA/2J mice. Retinas were obtained from DBA/2J mice of different ages (3, 6 and 11 months). C57BL6 mice were used as controls. As measured with HPLC, KYNA content decreased ($p < 0.01$) in the retinas of 6-month-old DBA/2J mice and continued to decrease ($p < 0.0074$) in the retinas of 11-month-old animals compared to the controls. Immunohistochemistry showed that expression of both KAT I and KAT II decreased markedly in the retinas of 11-month-old DBA/2J mice compared to controls. The impairment in KYNA biosynthesis in the retinas of DBA/2J mice may be one of the mechanisms of retinal neurodegeneration related to ocular hypertension.
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Keywords: Kynurenic acid; Kynurenine aminotransferases; Glaucoma; Retinal degeneration; DBA/2J mice

1. Introduction

The tryptophan metabolite kynurenic acid (KYNA) is the only known endogenous broad spectrum antagonist of all subtypes of ionotropic glutamate receptors (Perkins & Stone, 1982). It is preferentially active at the glycine allosteric site of the *N*-methyl-D-aspartate (NMDA) receptor (Birch, Grossman, & Hayes, 1988). It has been also demonstrated that KYNA is a noncompetitive antagonist of α7 nicotinic receptor (Hilmas et al., 2001).

KYNA has been detected in urine, serum, amniotic fluid (Milart, Urbanska, Turski, Paszkowski, & Sikorski, 1999), cerebrospinal fluid (Swartz, Matson, MacGarvey, Ryan, & Beal, 1990), and brain tissue (Swartz et al., 1990; Turski et al., 1988). Recently, KYNA has been identified and quantified in the rat and chicken retina (Rejdak et al., 2001, 2002) in concentrations similar to those observed in the rat, rabbit and human brain (Moroni, Russi, Lombardi, Beni, & Carla, 1988; Turski et al., 1988).

In the mammalian brain, KYNA is formed by irreversible transamination of L-kynurenine by kynurenine aminotransferases (KAT I and KAT II) (Guidetti, Okuno, & Schwarcz, 1997). KAT I has been localized immunohistochemically in the rat brain, medulla and spinal cord (Du et al., 1992; Kapoor, Okuno, Kido, & Kapoor, 1997; Knyihar-Csillik, Okuno, & Vecsei, 1999;

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Roberts, Du, McCarthy, Okuno, & Schwarcz, 1992). KAT II was first identified by Northern Blot mRNA analysis in the human brain (Okuno, Nakamura, & Schwarcz, 1991).

Immunohistochemical experiments using KAT I and KAT II antibodies (Okuno et al., 1991; Okuno, Tsujimoto, Nakamura, & Kido, 1993) showed that both enzymes are present in the rat inner retina. KAT I is preferentially expressed in Müller cell endfeet, while KAT II is localized in cells within the ganglion cell layer (Rejdak et al., 2001). Additionally, developmental changes of KYNA content in both vascularised rat and avascular chicken retinas during ontogeny have been reported (Rejdak et al., 2002). Moreover, our recent study also showed ontogenic changes of KAT I enzymatic activity in the chicken retina (Rejdak et al., 2003b). Results of both studies suggested a neuromodulatory role of KYNA in retinal ontogeny (Rejdak et al., 2002; Rejdak et al., 2003b).

Investigations of KYNA are of importance because KYNA has been shown to prevent neuronal damage caused by hypoxia or ischemia (Andine et al., 1988). It has been suggested that KYNA is involved in the pathophysiology of several brain disorders, e.g. Parkinson's disease (Ogawa et al., 1992), Huntington's disease (Beal, Ferrante, Swartz, & Kowall, 1991a; Beal et al., 1991b; Guidetti, Reddy, Tagle, & Schwarcz, 2000), and epilepsy (Yamamoto, Murakami, Horiguchi, & Egawa, 1995). Recently, it was suggested that KYNA deficiency is causally related to the pathology of excitotoxic retinal diseases and that NMDA-induced retinal ganglion cell (RGC) loss may cause alterations of KYNA content in the rat retina (Rejdak et al., 2003a).

The loss of RGC is a hallmark of many ophthalmic diseases, including glaucoma, retinal ischemia and optic neuropathy (Dreyer, Pan, Storm, & Lipton, 1994). The mechanisms of RGC death in those disorders are multifactorial, with a number of known risk factors (Quigley, 1998). The DBA/2J mouse is a promising animal model of ocular hypertension (John et al., 1998; Schuettauf, Quinto, Naskar, & Zurakowski, 2002). Mice of this strain spontaneously develop essential iris atrophy, pigment dispersion, and glaucomatous changes over time (Chang et al., 1999; John et al., 1998). Pigment dispersion and iris stroma atrophy are caused by distinct mutations in genes encoding melanosomal proteins (Anderson et al., 2002). Importantly, Schuettauf and colleagues reported a continuous loss of RGC during aging in this strain of mice (Schuettauf et al., 2002). The authors have also found that this murine glaucoma model is responsive to pharmacological treatments: RGC loss was blocked by the glutamate antagonist memantine given intraperitoneally, and by the β-blocker timolol applied as eye drops.

Since dysfunction of KYNA synthesis in the brain may be an important factor contributing to neuronal degeneration (Foster, Vezzani, French, & Schwarcz, 1984; Schwarcz et al., 1992), and NMDA antagonists prevent RGC loss in DBA/2J mice (Schuettauf et al., 2002), we propose that KYNA and its synthesizing enzymes may have relevance for the mechanisms of retinal degeneration in DBA/2J mice.

To verify this hypothesis, we examined changes of KYNA content and KAT I and KAT II cellular expression in the retina of DBA/2J mice during aging.

2. Methods

2.1. Animals

All experiments were performed in compliance with the guidelines of animal care in the European Community and the Association for Research in Vision and Ophthalmology. Female DBA/2J mice were housed with acidified water and special chow (ssniff M, Sniff Spezialdiäten, Soest, Germany), and a 24 h light/dark cycle was maintained.

2.2. Tissue preparation

Retinas from female DBA/2J mice at 3, 6 and 11 months were used in this study. Retinas from female C57BL6 mice were used as age-matched controls. Animals were sacrificed with CO_2, and the eyes were enucleated immediately. Following hemisection of the eyes along the ora serrata, the corneas, lenses and vitreous bodies were removed. To assay KYNA concentration, whole neural retinas were dissected free from the retinal pigment epithelium, choroid and sclera. Retinas were frozen immediately in liquid nitrogen after removal. For immunohistochemistry, the eyecups were immersion-fixed for 30 min in 4% (w/v) paraformaldehyde in phosphate buffer (PB; 0.1 M pH 7.4) at 4 °C. After washing in PB, tissues were cryoprotected by immersion in 30% (w/v) sucrose in PB overnight at 4 °C. Samples were then embedded in cryomatrix (Leica, Heidelberg, Germany).

2.3. HPLC

KYNA levels were investigated according to the method of Turski and colleagues (Turski et al., 1988). Specimens were sonicated in 2 vols (w/v) of distilled water, immersed in a boiling water bath for 10 min and centrifuged (10 min, 20 000 rpm). The resulting supernatant was diluted (1:1) with 0.2 N HCl and applied to a Dowex 50-W hydrogen form pre-washed with 0.1 N HCl. Columns were subsequently washed with 1 ml 0.1 N HCl and 1 ml water. KYNA was eluted with 2 ml of water. The elute was subjected to HPLC and KYNA was detected fluorimetrically according to the method of

Shibata (1988). HPLC reagents used in the study were obtained from Baker (Griesheim, Germany) and were of the highest available purity. Statistical analysis was performed using the unpaired Student's *t*-test.

2.4. Immunohistochemistry

Embedded eye cups were cut radially in 10–12 μm sections using a cryostat, collected on gelatine-coated slides, air dried, and stored at −20 °C for further use. Sections were incubated for 1 h with 20% normal goat serum (NGS; Sigma, Munich, Germany) and 0.3% Triton X-100 in phosphate-buffered saline (PBST) to reduce background staining. The primary antibodies were diluted in PBST containing 20% NGS + 2% bovine serum albumine (Sigma). For immunohistochemistry, sections were incubated with KAT I or KAT II antibody diluted 1:50 overnight at 4 °C. KAT I polyclonal antibody was raised in rabbits against rat kidney KAT I (Okuno et al., 1991), and KAT II polyclonal antibody was raised in rabbits against human liver KAT II (Okuno et al., 1993). An anti-rabbit secondary antibody (Molecular Probes) conjugated to Alexa 568 and diluted 1:1000 was used to mark the immunoreaction of KAT I and KAT II antibodies. Controls were made by omitting the first antibody.

3. Results

3.1. HPLC

KYNA concentrations in the mouse retina were in the same range as that observed in rats and chickens (Rejdak et al., 2001).

The KYNA content in the retinas of 3-month-old DBA/2J mice was 99 ± 6 pmol/g wet weight (mean ± SE; $n = 9$) and similar to that of the controls (109.5 ± 12; $n = 8$). In the retinas of 6-month-old DBA/2J mice it was found to be significantly lower ($p < 0.01$) compared to controls, 75 ± 7 ($n = 8$) and 108.7 ± 9 ($n = 8$) respectively. KYNA levels decreased at later ages in comparison to those in the C57BL6 mice ($p < 0.01$), reaching a concentration of 53 ± 10 in the retinas of 11-month-old animals (Fig. 1).

3.2. Immunohistochemistry

Immunoreactivity of both KAT I and KAT II was present in the inner retinas of DBA/2J mice as well as in those of the controls at the age of 3 months (Fig. 2A1, A2, C1, C2). KAT I immunoreactivity was mainly concentrated vitread the cell bodies in the ganglion cell layer in elements showing the morphological characteristics of Müller cells endfeet (see also Rejdak et al., 2001; Rejdak et al., 2003b) (Fig. 2A1 and A2). KAT I

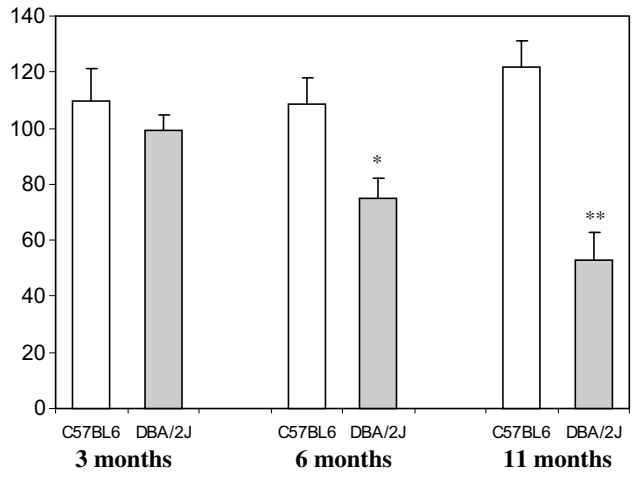

Fig. 1. Changes in KYNA contents in the retinas of DBA/2J mice at the ages of 3, 6 and 11 months in comparison to age-matched controls. Each column represents the mean KYNA concentration (pmol/g wt wet ± SE). White column—control, black—DBA/2J mice. (*) Significantly different KYNA content at 6 and 11 months when compared to the controls ($p < 0.05$ for each) by unpaired *t*-test.

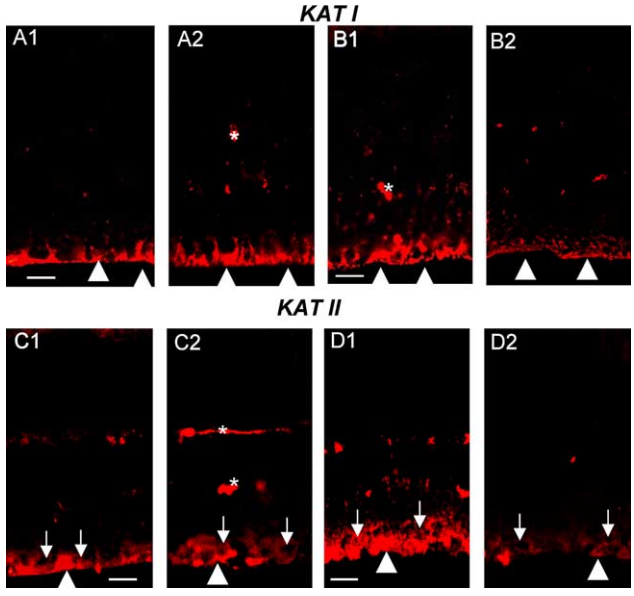

Fig. 2. Immunoreactivity of KAT I and KAT II in radial sections of the DBA/2J mice retina. KAT I is expressed in Müller cell endfeet (triangles = Müller cell endfeet). Intensity of KAT I immunoreaction is the same in 3-month-old controls (A1) and DBA/2J mice (A2). At the age of 11 months, retinal KAT I expression in control mice is the same as in 3-month-old animals (A1, B1); however, immunoreactivity is clearly reduced in 11-month-old DBA/2J mice (B2). At the age of 3 months, KAT II is expressed in the Müller cell endfeet (white triangles) and cell bodies within the GCL (arrows) in the retinas of both DBA/2J mice (C2) and the controls (C1). Intensity of KAT II immunoreaction is the same in 3-month-old controls (C1) and DBA/2J mice (C2). At the age of 11 months, KAT II expression in control mice is the same as in 3-month-old animals (D1, C1) but is clearly reduced in 11-month-old DBA/2J mice (D2). White asterisks indicate unspecific staining of vessels. Scale bar = 20 μm.

cellular expression was clearly decreased in the retinas of 11-month-old DBA/2J mice (Fig. 2B2) but not in their age-matched controls (Fig. 2B1).

KAT II was expressed with similar intensity in cells in the ganglion cells layer of both DBA/2J mice and controls at the age of 3 months (Fig. 2C1 and C2). These results were in agreement with our previous data obtained from adult rat retina (Rejdak et al., 2001). In parallel to KAT I, a decrease of retinal KAT II immunoreactivity was observed in 11-month-old DBA/2J mice when compared to their age-matched controls (Fig. 2D1 and D2).

4. Discussion

With their spontaneously developing ocular hypertension and time-dependent RGC loss, DBA/2J mice are a promising animal model for studying the consequences of chronic elevation of IOP (Schuettauf et al., 2002).

The present study demonstrated that parallel to this time-dependent RGC loss, KYNA concentration changes in the retinas of DBA/2J mice during aging. Retinal KYNA contents found in both C57BL6 and DBA/2J mice in early stages (3 months) were in the same range as those observed in rat and chicken retinas (Rejdak et al., 2002). The presence of KAT I and KAT II in the inner retina in both strains of mice was also demonstrated.

In retinas of 3-month-old DBA/2J mice, KYNA concentrations were similar to those observed in the control animals. A marked decrease of KYNA level was observed in retinas of 6-month-old animals, and it had decreased to 60% of control values by the 11th month of life. In contrast, the level of KYNA in C57BL6 mice did not change in 3-, 6- and 11-month-old animals.

It is now assumed that KYNA contents reflect its synthesis, since its storage in the brain has not been documented (Turski, Gramsbargen, Traitler, & Schwarcz, 1989).

In 1998, John et al. documented that the development of anterior segment anomalies resulted in an increase of intraocular pressure (IOP), causing the death of retinal ganglion cells, optic nerve atrophy, and optic nerve cupping in DBA/2J mice. The prevalence and severity of these lesions increased with age. The retinas of young 3-month-old DBA/2J mice had normal morphologic features that were similar to those observed in other mouse strains. As glaucoma developed, the nerve fiber layer became thinner. The ganglion cell layer (GCL) was the most susceptible to cell loss, which appeared to increase with age. In advanced cases the number of physiologically appearing ganglion cells was greatly depleted (John et al., 1998). Recently, it has been documented that this cell loss in a DBA/2J mice is age-dependent and starts in the sixth month of life (Schuettauf et al., 2002). By 11 months, optic nerve atrophy and disorganization were evident, and in 19-month-old DBA/2J optic nerves there was a marked reduction in the number of nerve fibers (John et al., 1998). According to Savinova et al. (2001), IOP values in the DBA/2J mice at the age of 2–6 months are approximately 12 mmHg (during the day) and approximately 14 mmHg (during the night), which is markedly lower than the values found in a number of other murine strains which do not develop glaucoma or any other ocular abnormality (including, *inter alia*, CBA/CaJ mice with the highest IOP values of all strains tested, >20 mmHg). RGC loss in 6-month-old and younger DBA/2J mice may therefore, precede the rise in the intraocular pressure.

Comparing our data with those demonstrated by other authors we found that KYNA was decreased by 24% from age 3 months to age 6 months, and by 46% until age 11 months. Schuettauf and colleagues found that RGC numbers decreased by 16% from age 3 months to age 6 months and by 56% until age 9 months (Schuettauf et al., 2002). Also, NFL thickness decreased in older DBA/2J mice but authors did not include measurements on NFL thickness, just qualitative statements without clear age correlation (John et al., 1998).

Since it has already been suggested that KYNA deficiency is causally related to the pathology of excitotoxic brain diseases (Schwarcz, 1992), the decrease in retinal KYNA synthesis might be considered as a contributing factor in the mechanisms of RGC loss, independently of increased IOP.

In agreement with our HPLC findings, the present study also demonstrated that cellular expression of both KAT I and KAT II in the inner retina decreases more during the aging of DBA/2J mice than in control animals. Since immunohistochemical studies have shown a preferential localization of KAT I and KAT II on Müller cell endfeet and RGC respectively (Rejdak et al., 2001; Rejdak et al., 2004), it appears that KAT malfunction leads to KYNA loss and this leads to one part of the RGC loss. Later in age, the process of RGC death exceeds the decrease of KYNA, and percentage of RGC loss is bigger than the reduction of KYNA production.

Glutamate has been shown to diminish KYNA synthesis, and it has been suggested that it has a regulatory influence on endogenous KYNA content (Urbanska, Kocki, Saran, Kleinrok, & Turski, 1997). Since that time, reports of increased glutamate in the vitreous in spontaneous disorders similar to glaucoma in dogs (Brooks, Garcia, Dreyer, Zurakowski, & Franco-Bourland, 1997), and quail (Dkhissi et al., 1999), and in the aqueous of rats after optic nerve crush (Yoles & Schwartz, 1998) have been published. It is conceivable that the decrease of KYNA synthesis in the retina of DBA/2J mice during aging observed in our study may be the result of increased glutamatergic transmission, but this remain to be determined.

Endogenous KYNA levels in the brain vary profoundly not only in response to a lesion but also during and after seizure activity (Wu & Schwarcz, 1996), as a result of energy deprivation, and in dystonia (Richter, Loscher, Baran, & Gramer, 1996). In several of these instances, these changes may affect the function of glutamate receptors.

The endogenous glutamate antagonist KYNA has been shown to prevent excitotoxic neuronal damage (Andine et al., 1988; Foster et al., 1984). Neuroprotective properties of KYNA are usually explained by its ability to block glutamate receptor functions. It has been found that an increase in brain KYNA concentration either directly, or through enhanced KYNA synthesis may be neuroprotective. In experimental brain ischemia neuroprotection has been observed following systemic administration of KYNA (Andine et al., 1988), L-kynurenine (Nozaki & Beal, 1992) as well as kynurenine-3-hydroxylase inhibitors (Cozzi, Carpenedo, & Moroni, 1999; Moroni, Cozzi, Peruginelli, Carpenedo, & Pellegrini-Giampietro, 1999). Importantly, systemic L-kynurenine administration partially protects against NMDA-induced degeneration of brain neurons (Nozaki & Beal, 1992) and retinal ganglion cells, and reduces visual discrimination deficits in adult rats (Vorwerk, Kreutz, Dreyer, & Sabel, 1996).

In conclusion, KYNA content decreases in the retinas of DBA/2J mice during aging. This phenomenon is accompanied by a decrease of KAT I and KAT II cellular expression. Our results suggest that KYNA deficiency and decreased cellular expression of its synthesizing enzymes may have relevance for the mechanisms of retinal degenerative diseases such as glaucoma or optic neuropathies. Further studies are needed to determine whether the decrease in retinal KYNA synthesis is the cause or the effect of neurodegeneration in the retina.

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